Reference: *Biol. Bull.*, **160:** 199–227. (April, 1981)

CIRCADIAN TIMING BY ENDOGENOUS OSCILLATORS IN THE NERVOUS SYSTEM: TOWARD CELLULAR MECHANISMS

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Abstract

The basic properties of circadian rhythms, such as oscillator type, entrainment to daily light-dark (LD) cycles, temperature compensation of the period length, and free-running periodicity, are remarkably similar in eucaryotic organisms from unicells to man. This encourages the view that all circadian oscillators are based on identical principles, found at the cellular level. Animals have a multioscillator organization, with brain centers (suprachiasmatic nuclei, optic lobes, etc.) and related structures (pineals, eyes, etc.) as sources of rhythmicity and coordination. Oscillators and driven activities are coupled by secretion (*e.g.* eclosion hormone, melatonin) or by direct neuronal connection. Oscillators of the multioscillator systems also are coupled. The cellular requirements for the circadian oscillator appear to be as generally uniform among various organisms as the basic properties. Ions and ion transport are important in the timing mechanism, as is protein synthesis on the eucaryotic ribosome. Although no concise model of the circadian oscillator encompassing protein synthesis, ions, and membranes has been offered, progress in analysis of the mechanisms has been made by genetic selection, screening of biochemical mutants, organ and tissue culturing, biochemical isolation of components, and chemical-pulse probing of the cellular oscillator.

Introduction

Anyone familiar with the cellular-level regulation of biological activities, *e.g.* insulin secretion, must be impressed with the excellent regulation of the complex

Non-standard abbreviations: CAP, compound action potential; CT, circadian time; DD, constant darkness; ERG, electroretinogram; LL, constant light; LD, light-dark cycle; NAT, N-acetyltransferase; PRC, phase response curve; SCN, suprachiasmatic nuclei.

processes involved. The more one knows about this regulation the more remarkable it seems. One thread (or more) of the web of regulatory processes in the cell is the endogenous circadian (about a day) clock. This clock controls overt cyclic activities in virtually all eucaryotes, from unicells to man. It is firmly established that circadian rhythms are a consequence of cellular oscillators. These oscillators are coupled to, and drive, such diverse activities as plant leaf movements, wheel-running in rodents, perch-hopping by birds, quantum catch of photoreceptors, and luminescent flashing of dinoflagellates. The precision of some of these overt rhythms reflects the precision of the underlying oscillator. For example, free-running locomotor activity in nocturnal rodents may show a standard deviation of 0.1 h (or 0.3%) out of a 23.9 h cycle period (Pittendrigh and Daan, 1976a).

Daily biological rhythms have been known for centuries. They were the subjects of enlightened enquiry by the astronomer De Mairan (1729) in the 18th century. They attracted the attention of eminent biologists such as Charles Darwin (1881). But only comparatively recently has the term circadian (coined by Halberg, 1960) come into common use in reference to a specifically defined phenomenon (Aschoff, 1965), and a vigorous search for the cellular basis of circadian rhythms begun. Approaches to discovering the mechanisms were considered in detail at the Dahlem Conference (Hastings and Schweiger, 1976). The present paper reviews the state of the search for circadian clock sites in animals, and for cellular and molecular bases of the circadian oscillator. This cellular emphasis complements other reviews (Block and Page, 1978; Rusak and Zucker, 1979) of the nervous system's participation in circadian rhythms.

Daily changes in environmental lighting and temperature result from the earth's rotation around its axis, and seasonal changes in these variables result from the earth's tilt as it orbits the sun. These fluctuating environmental stimuli strongly influence activities of plants and animals, resulting in driven activities that change with exactly the periodicity of the earth's rotation (24 h). In the absence of solar-day cues (*e.g.* in constant darkness and temperature) many of these activities continue to be periodic, but the cycles are only *about a day* (circa-dian). Periods of 21–26 h are common. This persistent cycling is the primary defining characteristic of circadian rhythms. Rhythms that require periodic stimuli, that do not continue in constant conditions, and that therefore lack an endogenous oscillator, are by definition not bona fide circadian rhythms. A complete definition (Hastings *et al.*, 1976), includes, among other things, compensation for temperature in the period length, and entrainment of the rhythms by environmental time-givers, such as light and temperature.

Since the characteristics of rhythms in unicells and man are nearly identical, circadian rhythms are thought to have been conserved during evolution, and to be of adaptive consequence. The biological usefulness of rhythms may be appreciated by considering the four categories of biological time measured by circadian clocks (listed by Pittendrigh, 1976): (1) programming a daily sequence of metabolic and behavioral changes, (2) enabling an animal to recognize a specific time of day and to return at that time on subsequent days, (3) enabling maintenance of a constant compass heading using the sun's azimuth as reference, and (4) making it possible to distinguish different durations of light and darkness as a measure of season (photoperiodism).

Entrainment and coupling

The circadian timer has an inherent period of about a day. It is brought into exact conformity with the solar day's environmental changes by the process of

entrainment, which advances or delays the phase of the endogenous oscillator at times in its cycle when it is sensitive to an environmental time-giving stimulus. The primary time-giver stimulus is light, although other stimuli also are effective in some systems. Typically, circadian clocks are most sensitive to light during the subjective night, the time in the circadian cycle when night would occur if the organism were exposed to a LD cycle. During early subjective night, light delays the phase of later cycles. During late subjective night, light advances the phase of latter cycles. The result is stable entrainment of the oscillator to a light-dark (LD) cycle, such as the solar LD cycle (Pittendrigh, 1974). The circadian oscillator's responses to light pulses at different phases of the cycle can be plotted against phase in the cycle to obtain a phase response curve (PRC). A PRC describes the underlying oscillator (Pittendrigh, 1974), since it characterizes the succession of points in the cycle in terms of sensitivity to the time-giver. As will be discussed below, the PRC obtained by responses to chemical agents is also a useful way to describe the effects of those agents on the timing mechanism.

Measuring the locomotor activity of a hamster in a running-wheel convinces one of the precision and reliability of circadian rhythms. But it leaves in question the driving oscillator's location and the mechanism that couples it to overt activities. Some answers have been gained by various techniques; creating lesions in specific tissue, isolating tissue within the organism, removing and then replacing tissue, and isolating tissue outside the organism, in organ or cell culture.

Many unicells have special advantages for particular analytical approaches. Therefore, the organization of rhythms in two such organisms, *Gonyaulax* and *Acetabularia*, will be examined before considering the complexity of metazoan rhythms.

The dinoflagellate *Gonyaulax*, has rhythms in photosynthetic capacity, cell division, glow, and luminescence, among others (Fig. 1A). These rhythms have different phase relationships in the circadian cycle. But all appear driven by the same basic oscillator, because they maintain a constant phase relationship and periodicity in free-running conditions, and change in phase to the same extent after a time-giver stimulus (McMurray and Hastings, 1972). Krasnow *et al.* (1980) recently examined their spontaneous light-emitting rhythm. It consists of a glow rhythm and a flashing rhythm. The phase difference between these two changes with time in constant conditions and depends upon light intensity. This raises the possibility of control by more than one oscillator.

The giant single-cell alga, *Acetabularia*, has several measurable rhythms, including O_2 production, chloroplast migration, and extra-cellular electrical currents. With its nucleus removed, the cell will survive and still have good rhythms. Since rhythms in unicells are usually measured on populations in culture, it has been asked if each cell is capable of expressing a rhythm in isolation, and to what extent individuals in a population interact in generating a rhythm. Individual cells of *Acetabularia* (Karakashian and Schweiger, 1976a) or *Gonyaulax* (Sweeney, 1960) apparently express a circadian rhythm. Individuals of a population interact very little, judging by the spread in the distribution of period lengths on successive days of free-running activity.

The *Gonyaulax* rhythms shown in Figure 1 illustrate the general concept that several activities may be coupled to the basic cellular oscillator. The observable rhythmic activities are quite separate from the basic oscillator, but they serve as indicators (or "hands") of the clock's performance. Selectively suppressing one of these activities does not perturb the clock (Hastings, 1960). This suggests that the

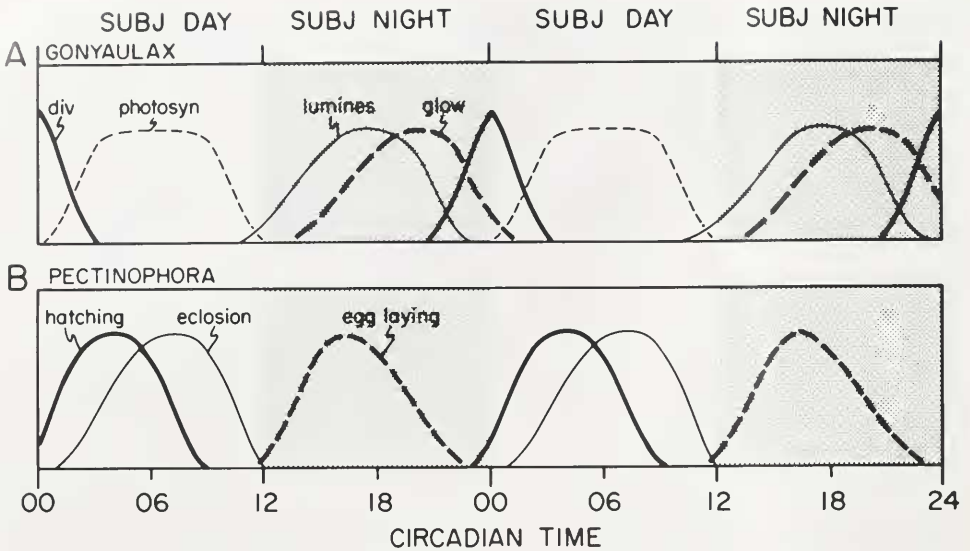

FIGURE 1. Overt rhythmic activities coupled to the circadian clock. In A, the four separate activities of cell division (div.), photosynthetic capacity (photosyn.), bioluminescence (lumines) and glow exhibited by a *Gonyaulax* culture are shown. All four are coupled to the same cellular clock, since they maintain a fixed phase relationship. B shows three separate activities, egg hatching (hatching), egg laying, and pupal eclosion, exhibited by the moth *Pectinophora* at separate stages in the life cycle. The curves in B are the distribution of events for a population of organisms. Two days of circadian time in hours are shown, divided into subjective day and subjective night. Redrawn from McMurray and Hastings (1972) and Pittendrigh (1976).

activities themselves do not influence the mechanism by feedback and are, therefore, not part of the circadian timing device.

Activities that occur only once in the organism's lifetime, as well as ongoing daily activity rhythms, are coupled to the basic oscillator. An example among metazoans is the moth *Pectinophera*, where the circadian clock times egg laying, egg hatching, and pupal eclosion, which occur at different stages of the life cycle and different phases of the circadian cycle (Pittindrigh, 1976; Fig. 1B). Members of a moth population will perform these activities with a temporal distribution like that shown in the figure. For example, some moths will go through eclosion on one day at a certain time of day. Other moths may do it on the next day, but always at the appropriate time.

Multioscillator organization in metazoa

If each unicellular organism has a circadian oscillator, each cell in a multicellular organism may have at least the potential for it. But instead of each metazoan cell going its separate way, specific sites, arranged hierarchically, appear to be important in generating rhythms. Intact animals kept under constant conditions, so that circadian rhythms are free-running, reveal both the multioscillator nature of the circadian organization, and interactions between oscillators. In man, activity-rest cycles proceed with the same periodicity as other rhythms of ion excretion, body temperature, etc. However, after some days without time cues, these rhythms may break away from one another (Fig. 2) and continue at their own periodicity

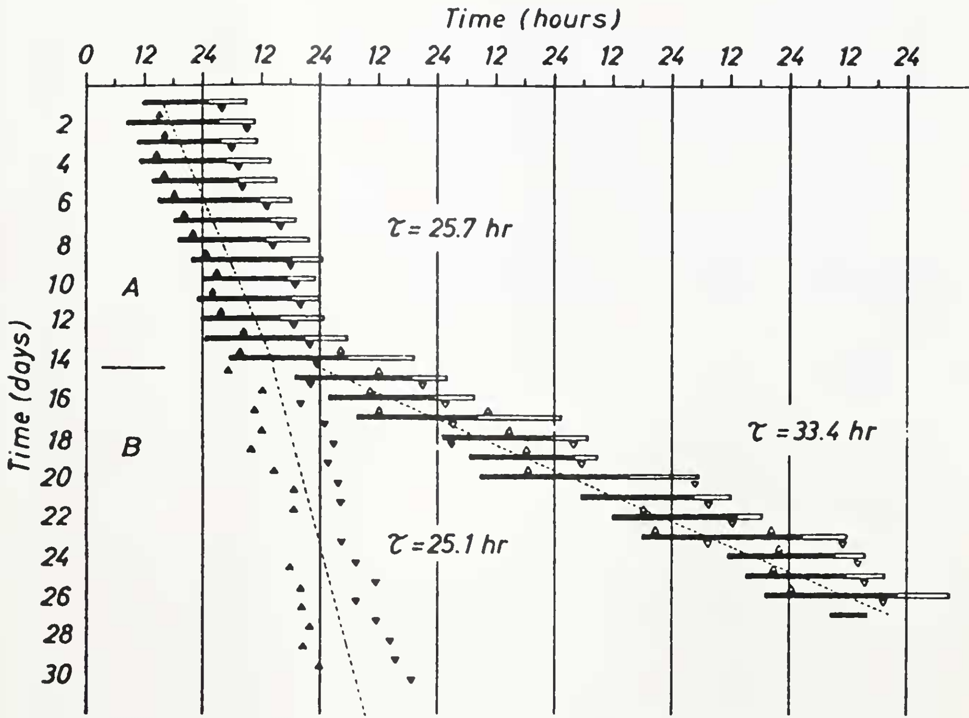

FIGURE 2. Rhythms of a human subject under constant conditions without time cues. Successive days of activity are plotted downward against a scale of solar time. The bars are activity (black) and rest (white). The triangles are maxima and minima of rectal temperature. The free-running periods (τ) are 25.7 h before the spontaneous desynchronization (A) and 33.4 h for activity and 25.1 h for temperature afterwards (B). From Wever (1975).

(Aschoff and Wever, 1976; Wever, 1979). This internal desynchronization shows the existence of several circadian pacemakers usually coupled to one another. Figure 2 shows an example in which activity and rectal temperature were proceeding at the same periodicity, 25.7 h. After desynchronization, each rhythm continued with a different period (33.4 h and 25.1 h) than the previous 25.7 h period, suggesting that the clocks were interacting.

Another phenomenon observed in mammals kept under prolonged constant conditions is "splitting." The activity-rest cycle proceeds with a normal free-running periodicity for some time and then abruptly splits into two activity bands instead of one, the two adopting a 180° anti-phase relationship. This again is evidence for more than one pacemaker. To account for "splitting" and other circadian phenomena, Pittendrigh and Daan (1976b) proposed a circadian system having two kinds of oscillators, with opposite dependencies.

Something similar to "splitting" has been observed in the rhythms of neuronal activity in *Aplysia* eyes. Each eye has the complete organization of a circadian clock. If intact animals are entrained to light-dark cycles, the two circadian clocks adopt identical phases. However, if the animal is placed in constant dim light for days, the eyes adopt different phases (Hudson and Lickey, 1980). The tendency toward 180° anti-phase relationship similar to the "splitting" observed in mammals suggests weak coupling between the separate clock sites.

The Brain–Clock Connection

Discrete tissues devoted to the clock function profoundly affect overt circadian activities. In animals, these tissues are brain centers or related structures commonly associated with light reception and neurosecretory activity.

Gastropod eyes

The "sea hare," *Aplysia*, is an example of gastropod circadian organization whose cellular and hierarchical organization has been studied in considerable detail. *Aplysia* became a recognized model for circadian studies with the pioneering work of Strumwasser (1965) on a single identifiable neuron. Intracellular recordings of membrane electrical activity seemed to show a bona fide circadian rhythm, but later tests showed that the neuron failed to sustain a circadian rhythm (Beiswanger and Jacklet, 1975; Lickey *et al.*, 1976). This discouraged the use of that single neuron as a suitable experimental preparations, and leaves in doubt the existence of any circadian rhythm in it. A single neuron exhibiting a distinct circadian rhythm has yet to be found, but the idea of a cellular clock coupled to and driving membrane electrical activity was established.

Further study revealed a robust circadian oscillator system in the *Aplysia* eye (Jacklet, 1969a). Rhythm in electrical activity was recorded from the isolated eye-optic nerve. This established that the rhythm is generated by a discrete organ, and raised the possibility of testing the animal's circadian abilities in the presence and absence of this known clock. The eye of *Aplysia* is small and inconspicious, as perhaps befits an animal with limited visually oriented behavior. The eye, a closed-vesicle type, has a central lens surrounded by a complex retina of several thousand neurons and photoreceptors. The long optic nerve is convenient for electrical recordings, made continuously while the eye is maintained in controlled environmental conditions in an organ culture. The most conspicious activity recorded from the optic nerve is compound action potentials (CAP) which represent the synchronous firing of a population of neurons (Jacklet, 1969b, 1973). CAPs are evoked by light or occur spontaneously in darkness. Dark activity changes in a rhythmic way, as shown in Figure 3, and continues to do so for 2 weeks or more in isolation. The period of the rhythm depends upon the composition of the culture medium: In artificial seawater alone the period is 23–24 h. In a nutrient medium the period increases to 26–28 h (Jacklet, 1971), depending primarily upon amino acids present in the medium.

Figure 3 shows the circadian rhythm as recorded in constant darkness at 16°C. The time axis is in circadian time (CT), which refers to 24 circadian h for each cycle of the rhythm. In this case the period length is 26 h of solar time, so each circadian hour is equal to slightly more than one solar day hour. In circadian time (CT) the 00 hour refers to subjective dawn, the time when dawn would have occurred in the circadian cycle. Dusk is then 12 h, and 18 h is the middle of the subjective night. Using this notation, one can appreciate that electrical CAP activity normally begins before dawn (anticipation). This activity remains high during the subjective day and becomes low during the subjective night.

Endogenous CAP activity in complete darkness, sampled at several circadian times, is shown in Figure 3B. As activity commences at CT 22, CAPs occur at regular but long intervals. At CT 00, when the frequency is half its maximum rate, CAPs occur at shorter intervals and are clustered in "bursts" of two or three. At CT 02, the frequency is near maximum, activity is clustered into bursts of four, and the amplitude of the CAPs is more than twice that at CT 22. Activity is still high at CT 05 but diminished at CT 21. The activity subsides by decreases in the

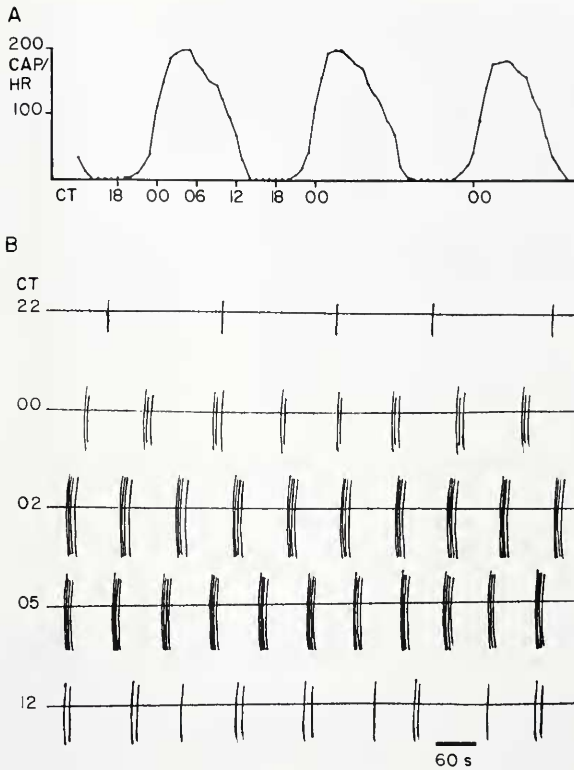

FIGURE 3. Circadian rhythm of endogenous compound action potential (CAP) frequency recorded from the optic nerve of an isolated *Aplysia* eye in organ culture during constant darkness. In A, the frequency is plotted against circadian time (CT). B shows the actual CAP record representative of each CT (22, 00, etc.). Note the changes in amplitude as well as frequency with advancing CT. The firing mode also changes, from pacemaking to "bursting." Largest CAPs are 100 μV and time scale is 60 s in B. Jacklet, unpublished.

number of CAPs per burst and later by increases in the interval between bursts, similar but in opposite sequence to the increases.

Intracellular circadian oscillators probably control the observed CAP firing, since the membrane CAP activity can be artificially suppressed or enhanced without changing the period or phase of the circadian rhythm (Jacklet, 1973; Eskin, 1977). The CAP pacemaker or "bursting" activity, which occurs at intervals of minutes, is produced by a separate membrane oscillator involving changes in ionic conductance. The circadian oscillator modulates the CAP "bursting" activity in a systematic way. One imagines the membrane conductances and/or ion transport properties of each CAP-generating neuron being altered over the circadian cycle by couplings to the intracellular circadian oscillator, with silence impressed at one phase and bursting pacemaker activity induced at the other.

In *Aplysia*, the active portion of the activity cycle corresponds to the active phase of locomotor activity. Simultaneous recordings of optic nerve activity and locomotor activity from intact, freely moving sea hares show that locomotor onset closely follows the CAP activity that anticipates dawn (Block, 1979). The eyes are not the critical photoreceptor for the diurnal locomotor activity of *Aplysia*, since eyeless animals remain diurnal on light-dark cycles at 200 lux, but do not anticipate

dawn (Lickey *et al.*, 1976). However, the eyes are an important influence when locomotor activity is allowed to free-run in the absence of a forcing LD cycle. Periodicity deteriorates in eyeless animals (Strumwasser, 1973), although some still have weak periodicity (Lickey *et al.*, 1977). It appears that the eyes are the major pacemakers for circadian locomotor activity, but that other weaker sources also influence this activity (Lickey and Wozniak, 1979; Strumwasser *et al.*, 1979).

Intact neural connections from the eye to the central nervous system are necessary in order for the eye to influence locomotor activity (Lickey *et al.*, 1976). This is true even though the eye secretes polypeptides (Harf *et al.*, 1976) and some of the peptides (~1000 MW) are released rhythmically in phase with the rhythm of CAP activity (Strumwasser *et al.*, 1979). The target of this secretion is not known.

The synchronous firing of retinal neurons (CAP activity) may efficiently release neurosecretory material (Jacklet, 1969b). This idea is supported by the finding that CAPs are produced by the secondary or D (dark) neurons, shown in Figure 4 and identified by dye injection and intracellular recording (Jacklet, 1976, 1979). These neurons have the ultrastructural characteristics of neurosecretory cells, including dense core vesicles (Luborsky-Moore and Jacklet, 1977; Strumwasser *et al.*, 1979).

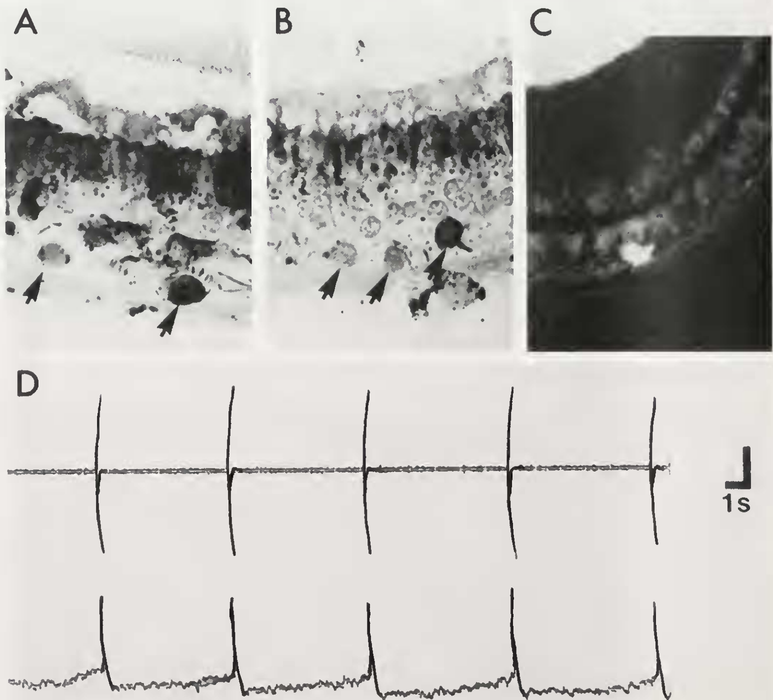

FIGURE 4. Output neurons of the circadian oscillator in the *Aplysia* eye. A, B, and C are histological sections of the retina showing receptors in the pigmented layer and secondary or D neurons (arrows) below. Some of the neurons (~15 μm in diameter) are backfilled with cobalt in A and B. The neuron in C was filled with Lucifer yellow, a fluorescent dye, by intracellular injection. Only the D neuron is filled with dye; other light areas of the retina are autofluorescence. In D, the endogenous activity from the optic nerve (top) and a simultaneously impaled "D" neuron (bottom) is shown. Time scale is 1 s and voltage is 20 μV, top, and 10 mV, bottom. From Jacklet and Schuster, unpublished.

Rhythmic CAP activity probably is responsible for the rhythmic polypeptide release shown by Strumwasser *et al.* (1979). Peptides or catechol amines (Luborsky-Moore and Jacklet, 1977) may be released at the terminals of the optic nerve in the cerebral ganglion, since ligation of the optic nerve leads to accumulation of fluorescent material on the eye side of the ligation. The eye contains serotonin and lesser amounts of dopamine. Pulses of exogenous serotonin phase shift the eye rhythm (Corrent *et al.*, 1978).

The circadian clock organization observed in *Aplysia* eyes is not characteristic of all gastropod eyes, but other examples are known. The eye of *Navanax* is similar to *Aplysia's* (Eskin and Harcombe, 1977): the optic nerve CAPs are driven in an endogenous rhythm. Comparable organization holds for the eye of *Bursatella*, the frilled sea hare, where the period of the rhythm is somewhat shorter (21 h compared to 24 h in *Aplysia*) and the effect on locomotion is less distinct (Block and Roberts, 1980).

Arthropod brains

The optic lobes of the cockroach are the site of the circadian clock controlling its locomotor activity, and its compound eye is the photoreceptor that mediates light entrainment of the oscillator (Brady, 1969). Lesions of the optic lobe implicate the lobula as the site (Roberts, 1974; Sokolove, 1975). An intact neural pathway from the optic lobes to the thoracic ganglia is necessary for expression of the rhythm. Therefore, the coupling is believed to be neural. In addition, attempts to repeat earlier studies showing humoral coupling failed (see Brady, 1969).

In crickets, three rhythms have been studied: locomotion, stridulation, and spermatophore production. The clock appears to be in the optic lobe. It is entrained via the compound eyes, and neural connections from the optic lobes to the brain are necessary. The pars intercerebralis serves as a coupling site between the optic lobe oscillator and the various behaviors. The channels from the pars intercerebralis may be neural or humoral (Sokolove and Loher, 1975).

Among insects, as among gastropods, clock sites and coupling mechanisms are not identical. In moths, the flight activity rhythm is controlled by a cerebral lobe clock, not the optic lobe, and entrained via extraocular receptors in the brain itself. It is neurally linked to the thoracic motor centers for flight (Truman, 1974). Contrasting with this arrangement is the moths' control of eclosion behavior. Again the clock is in the cerebral lobes and a brain receptor mediates entrainment by light. However, the coupling between timer and eclosion is via a humoral agent, the eclosion hormone. This arrangement was demonstrated by transplanting the brain from one animal into the abdomen of a debrained animal (Truman, 1972) and observing that eclosion occurred at times appropriate for the transplanted "loose brain" in the abdomen, and that the circadian phase of subsequent eclosion behavior was appropriate for the brain's clock. Thus, transplanting the brain clock transferred the phase. The eclosion hormone triggers programmed neuronal activity from the abdominal nervous system, which directs eclosion behavior (Truman, 1979).

Handler and Konopka (1979) recently demonstrated another case of humoral coupling, in *Drosophila.* Transplanting a brain from a clock-period mutant fly caused an arrhythmic host fly receiving the transplant to assume the periodicity expected of the transplanted mutant brain. Here, a humoral coupling, rather than the direct neural connection presumed for most other insects studies, apparently drives the circadian locomotor activity.

The eye of *Limulus*, the horseshoe crab, has been studied extensively from the standpoint of visual physiology and feature extraction. But only recently has it been recognized (Barlow *et al.*, 1977) that a circadian clock affects the eye's performance. Both electroretinogram (ERG) and optic nerve responses of the lateral eye change in magnitude in response to standard light pulses. Highest firing rates of optic nerve fibers are obtained at night, when efferent nerve activity from the brain to the eye is high (Fig. 5). The animal moves most at night. Normally, a circadian clock in the brain controls the rhythmic change in efferent activity, but selectively shocking the optic nerve with electrical pulses activates the efferent fibers and elevates the amplitude of the visual response. It appears, therefore, that the efferent fibers couple the clock output to the light receptors. This effect has been demonstrated in single retinula cells (Kaplan and Barlow, 1980). The efferent activity decreases the photoreceptor noise (quantum bumps) and increases the photoreceptor response (receptor potential) to light. The response increases partly because changes in the morphology of the retinula and surrounding pigment cells maximally expose the photosensitive rhobdom to light. But the mechanism for reducing noise is obscure.

The efferent activity controlled by the circadian oscillator also is responsible for enhanced turnover of the photosensitive membranes. Onset of first light, at dawn, causes synchronous breakdown and then reassembly of these membranes, but this process can be blocked by blocking the efferent optic nerve activity (Chamberlain and Barlow, 1979). The morphology of the ommatidial cells also changes under the influence of the circadian clock in the brain (Barlow *et al.*, 1980).

Crustaceans also show rhythmic changes in light responsiveness and locomotor activity. Aréchiga and Wiersma (1969) reported an interesting circadian rhythm (period 22–24 h) of electroretinogram amplitude and activity of single visual units (sustaining fibers) in crayfish. Sensitivities are highest during the nocturnal peaks in locomotor activities. The various rhythms observed are in phase and appear to

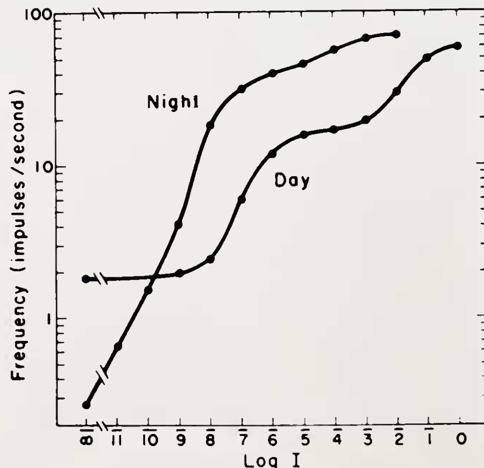

FIGURE 5. Differences in firing rates of a single optic nerve fiber of *Limulus* eye in response to standard test flashes. The animal was in continuous darkness between flashes. "Day" responses were recorded from 1500–1600 h and "night" responses from 2100–2200 h. Modulation by the circadian clock, carried by efferent fibers to the eye, is responsible for the shift in sensitivity at "night." Log I = 0 is 10^{12} quanta/s at cornea incident on the single ommatidium from 400–650 nm. From Barlow *et al.*, 1977, copyright 1977 by the American Association for the Advancement of Science.

be driven by a common clock mechanism and mediated by a humoral substance. Support for humoral mediation comes from experiments showing that injecting eyestalk extracts reproduces daytime phase characteristics (quietness) (Aréchiga *et al.*, 1974). The eyestalk factor, also found elsewhere in the nervous system, is proteinaceous and depresses neuronal activity—hence its name, neuro-depressing hormone. A variety of crustaceans may use the same substance, since there is cross-sensitivity among species (Aréchiga *et al.*, 1979).

The pineal of vertebrates

Pineal organs, which arise from evaginations of the diencephalon of the brain, are one class of circumventricular organs. Structure and innervation of pineals differ pronouncedly in lower and higher vertebrates (Wurtman *et al.*, 1968).

The pineal gland of the sparrow contains a circadian oscillator. When kept in constant darkness, sparrows deprived of their pineal glands become arrhythmic, and lack the periodic circadian locomotor activity of normal birds. However, pinealectomized sparrows appear to entrain to light-dark cycles rather than being directly driven by the cycles, since such birds anticipate lights-on. This suggests that other "damped" oscillators may directly drive locomotion. An alternative is that other oscillators (*e.g.*, the suprachiasmatic nuclei) depend upon the pineal for coupling their action to locomotion (Takahashi and Menaker, 1979). To know a sparrow is not to know all birds, however. Pinealectomy disrupts the system but does not cause permanent arrhythmia in starlings (Gwinner, 1978), and gallinaceous birds are not affected.

When Zimmerman and Menaker (1979) transplanted pineal glands into the anterior chamber of the eye of pineal-less birds, the recipient birds regained a circadian rhythm. Furthermore, the phase angle of the rhythm, measured from the onset of activity in the host animal after the transplant, was as expected if the transplanted pineal contained the clock. Thus, the transplantation did not significantly perturb the clock in the pineal, and the recipient bird's locomotor activity quickly became coupled to it, presumably by the pineal secretion melatonin.

The pineal of the chicken is similar: When it is isolated in organ culture, the circadian rhythm of melatonin and associated enzymes of the biochemical pathway persist (Binkley *et al.*, 1978). Melatonin is synthesized in the pineal by a well known biochemical pathway (Klein, 1974). The amino acid tryptophan circulating in the blood is converted to 5-hydroxytrytamine (serotonin) in the pineal. Serotonin is converted by the enzyme N-acetyltransferase (NAT) to N-acetylserotonin, which is acted on by hydroxyindole-o-methyl transferase (HIOMT) to produce melatonin. Melatonin is produced rhythmically by this pathway, with production highest during the dark and lowest during light. The two enzymes, HIOMT and especially NAT, also change in activity rhythmically. The strong rhythm in NAT activity seems largely responsible for the rhythmic changes in serotonin and melatonin concentrations.

Isolated perfused chicken pineal glands *in vitro* release melatonin rhythmically (Takahashi *et al.*, 1980). When individual pineal glands of 5–8-week-old chickens were placed in constant darkness, the rhythm continued, but with a reduced amplitude (Fig. 6). These experiments show the pineal has an endogenous circadian oscillator that may be coupled to other activities by melatonin secretion.

Deguchi (1979) maintained dissociated chicken-pineal tissue in a cell culture. He found that NAT activity from the dissociated pinealocytes was rhythmic: activity was highest in darkness and lowest in light. NAT activity persisted as an

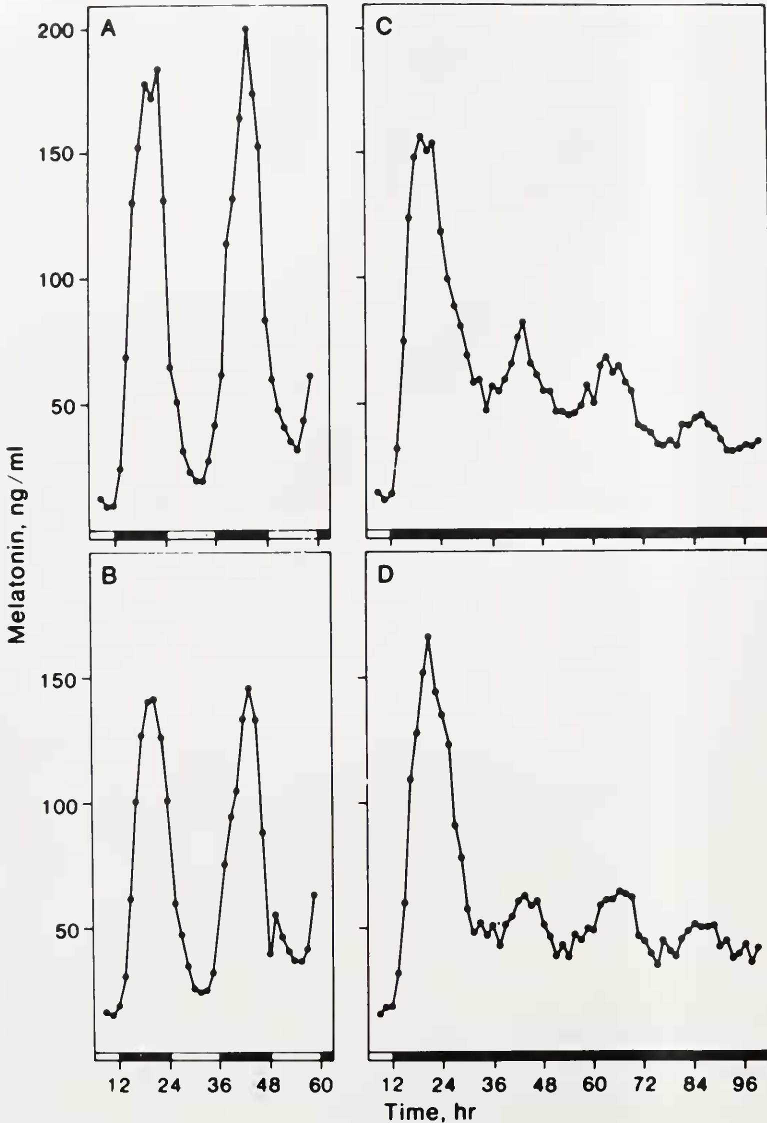

FIGURE 6. Rhythms of melatonin release from isolated perfused chicken pineals. A and B are in light-dark cycles and C and D are in constant dim red light (1–5 lux). Each record is from one pineal. In the constant dim light the rhythms persist, although damped, showing the presence of an endogenously timed release mechanism. From Takahaski *et al.*, 1980.

endogenous circadian rhythm in constant darkness. It entrained to a reversed photocycle. This establishes that the cell culture contains both a circadian clock and the photoreceptor that entrains the clock to light. Since the dissociated pinealocytes are not in an organized tissue, these results also suggest that each cell has its own photoreceptor and circadian oscillator, coupled to the enzyme NAT. Romero *et al.* (1975) blocked the rat pineal's rise in NAT activity in darkness with cyclo-

heximide, showing protein synthesis is necessary. RNA synthesis is also required at some times in the cycle.

The rat pineal also produces melatonin rhythmically, but the circadian oscillator is not in the pineal. Oscillators in the suprachiasmatic nuclei drive the pineal rhythm (Moore and Klein, 1974). The two are coupled via neural connections of the sympathetic nervous system. The neurotransmitter of the sympathetic system, norepinephrine, is released and combines with β-receptors on the pineal. This activation of the β-receptors causes c-AMP production, which promotes NAT synthesis. This forced rhythm of the rat pineal, driven by the suprachiasmatic nuclei (SCN), contrasts with the endogenous oscillator in the chicken pineal.

Melatonin is also synthesized in the retinas of many vertebrates. Recently, circadian rhythms of melatonin content and NAT activity have been shown in chick retina (Hamm and Menaker, 1980). These rhythms persist in constant darkness and are not abolished by pinealectomy. The levels of NAT and melatonin in the retina are similar to those found in the pineal. Light inactivates NAT activity, as it does in the avian pineal.

Melatonin rhythms may promote or modulate rhythms of shedding of outer segment disks from retinal rods. Melatonin rhythms also could affect pigment migration, and photomechanical movements of rods and cones.

The suprachiasmatic nuclei of vertebrates

Ablation identified the SCN of the mammalian brain as an important circadian oscillator (Moore and Eichler, 1971; Stephen and Zucker, 1972). These bilaterally symmetrical nuclei of the hypothalmus lie just above the optic chiasm (Fig. 7) and receive light information by a retinohypothalmic tract. They control mammalian rhythms such as locomotion and pineal secretion, and are near the top of a hierarchy of oscillators that control mammalian rhythms and integrate the activity of other suspected oscillators into a circadian framework (Rusak, 1979).

An improvement over SCN ablation uses a Halasz knife to surgically isolate, but not remove, an island of rat hypothalmic tissue containing the SCN (Inouye and Kawamura, 1979). Recordings inside the island and at neighboring hypothalmic sites showed that circadian neuronal activity persisted only inside the island.

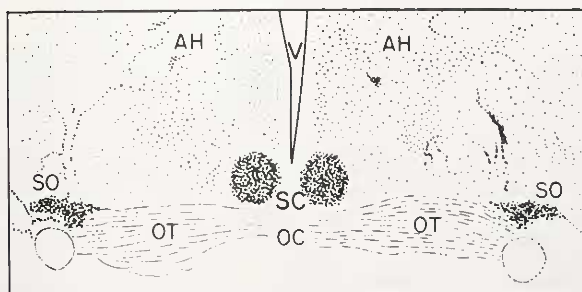

FIGURE 7. Suprachiasmatic nuclei of the rat are shown in a frontal section through the hypothalmus. The nuclei have been selectively ablated, isolated as an island of tissue, and studied by the deoxy-glucose method. These studies implicate them as circadian clock sites. Suprachiasmatic (SC) and supraoptic (SO) nuclei, optic chiasm (OC), optic tract (OT), anterior hypothalmus (AH) and third ventricle (V) are shown. Adapted from Moore and Klein, 1974; Inouye and Kawamura, 1979.

More distant sites, such as the raphe, substantia nigra, and reticular formation, also lost their rhythmicity. Normally, these rhythmically active brain structures are most active during projected night, when rats are most active. Some recordings from the hypothalmic island showed maxima at night but others had maxima at day. Later, histological examination of the brain recording sites showed that recordings with day maxima were from the SCN proper, while those with night maxima were from other sites within the island. The rhythmic activity in tissue outside the SCN is 180° inverted in phase compared to the SCN activity. The maximum SCN activity during projected day corresponds to the maximum 2-deoxy-D-glucose uptake in the SCN during projected day (Schwartz *et al.*, 1980). Thus, the SCN appears to be the site of an important circadian oscillator, coupled to surrounding brain structures primarily by neural connections.

Destruction of sparrows' SCN disrupts free-running locomotion, producing arrhythmia. The birds still entrain to LD cycles, so the results approximate those for pinealectomy (Takahashi and Menaker, 1979). These authors favor the idea that a third oscillator, with input from light receptors, can entrain locomotion.

While ablation helps identify a candidate structure, it cannot reveal the timing mechanism. One approach to the latter is to study the metabolism of brain structures. The autoradiographic 2-deoxy-D-glucose method has been used to measure rhythmic glucose utilization in the intact SCN of rats (Schwartz *et al.*, 1980). Glucose use should reflect the functional activity of the SCN (Schwartz and Gainer, 1977), since brain structures depend heavily upon glucose for energy. Animals were injected intravenously with radiolabeled 2-deoxy-glucose and killed 45 min later. Frozen sections of the brain were made, autoradiographs prepared, and glucose utilization calculated. Under an LD cycle, the SCN glucose utilization was rhythmic. It was highest during the light portion of the cycle, coincident with highest rates of neuronal firing. The rhythmic pattern persisted in prolonged darkness (Fig. 8) and after bilateral enucleation, showing that the rhythm is endogenous to the SCN and not simply driven by light input. Further questions about SCN mechanisms can be approached by refinements in this method. Some obvious questions are what proportions of the energy are used for ion pumping, macromolecular synthesis, and transmitter release.

Cellular and Molecular Mechanisms

Genetic selection

Genetic analysis and mutants have enabled investigators of complex biological systems to deal with a specific facet of a system, rather than analyze the total system's responses. This approach has been useful in dissecting the circadian-clock system. Two complementary approaches have been used. One involves isolating and characterizing clock mutants and attempting to analyze the primary gene products biochemically. The other approach is screening known biochemical mutants for circadian-clock abnormalities. This should identify important mechanisms in the circadian oscillator.

Several clock mutants are known. In *Drosophila*, a number have been isolated after induced mutagenesis. Konopka and Benzer (1971) used ethyl methane sulfonate (EMS) to mutagenize male *D. melanogaster.* These males were mated with attached-X females to produce male progeny with identically mutagenized X chromosomes. Screening these flies for abnormal rhythms in eclosion and activity led to identification of three clock mutants, one with a long period, per^l, one with a

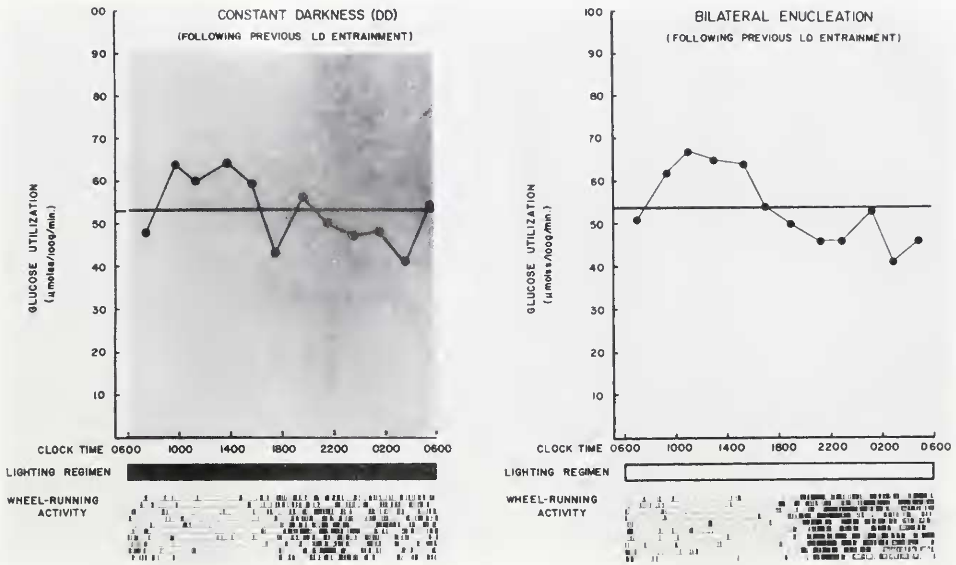

FIGURE 8. Glucose utilization by the SCN in normal rats in constant darkness (left) and bilaterally enucleated (right) rats in constant light. In both cases the utilization was rhythmic with highest points during projected day, even though locomotor activity is highest (bottom records) during projected night. Each point on the upper graphs represent one animal, except for 1100. From Schwartz *et al.*, 1980.

short period, per^s, and one that was aperiodic, per^0. All three mutants mapped to approximately the same location on the X-chromosome. Complementation tests showed that two are recessive to wild type, since heterzygotes that contain one normal and one mutant X-chromosome have nearly normal circadian rhythms. Gynandromorphs with clock-mutant X-chromosomes have been constructed (Hotta and Benzer, 1972) and behavioral mapping of these mosaic flies showed that the clock is in the brain, and that it probably exists independently on each side of the symmetrical brain.

In addition to altered periodicity, the mutants have altered responses to phase resetting after light pulses. Normal *D. melanogaster* have a small-amplitude phase-response curve (light shifts the oscillator in small steps). But the short period mutant has a phase-response curve with a larger amplitude, and a smaller portion of the cycle where light has no effect. So the mutations affect not only the period, but also other aspects of the circadian clock organization (Konopka, 1979).

Genetic clock mutants obtained in *Drosophila pseudoobscura* (Pittendrigh, 1974) have been studied by testing the clock control of the eclosion of pupae to adults. EMS mutagenesis similar to that performed in *D. melanogaster* produced five mutants that fall into two groups. Group 1 shows weak periodicity in a light-dark cycle and no periodicity in constant darkness. Group 2 shows no periodicity in either regimen. Mutants within each group do not complement one another, but partial complementation is obtained for heterozygotic flies with group 1 mutations on one X-chromosome and group 2 mutations on the other X-chromosome. They are periodic in constant darkness, but the phase and pattern of their rhythms are altered.

Since the *D. melanogaster* mutants map to the brain in genetic mosaics, it should be possible to transplant a fly brain to a mutant fly and have the transplant's

circadian rhythm expressed, provided the clock's output becomes coupled to the locomotor apparatus. This result has been obtained. Handler and Konopka (1979) transplanted brains from short-period mutant (per^s) animals to the abdomens of aperiodic-mutant (per^0) host flies. The hosts then expressed short-period rhythms. These experiments show that the clock is in the transplanted brain and is coupled to the locomotory control mechanism by a humoral agent, since no neural attachments were restored. Recent evidence suggests that neurosecretory cells in the brain are concerned with the clock, because these cells are located atypically in the aperiodic mutant (Konopka and Wells, 1980) and in the *D. pseudoobscura* mutants. The aperiodic mutation has a significantly larger percentage of neurosecretory cells at the top edge of the brain. However, normal morphological distribution of neurosecretory cells does not guarantee normal circadian rhythmicity, since a few per^0 individuals with normal cell morphology still show aperiodic locomotor activity. These neurosecretory cells probably are involved in the circadian clock system, perhaps releasing a humoral substance controlling locomotor activity (Konopka and Wells, 1980).

The photosynthetic flagellate *Chlamydomonas* has rhythms in phototaxis and growth. Most wild-type strains have normal circadian periods, but a wild-type strain was found with a period 3 h shorter than average (Bruce, 1976), showing that variation does occur in wild populations. Mutagenesis of normal strains with nitrosoguanadine produced long-period mutants (Bruce, 1972). The mutants behave like single-gene mutations, since pairwise crosses between mutants resulted in recombinant forms from all crosses. Double mutant recombinants indicate additive effects of the genes: double mutants' periods are lengthened by twice the single mutant increase of 2.5–4 h (Bruce, 1976). Analysis of three of these mutants, per-1, per-2, and per-4, demonstrated recessive, dominant, and incompletely dominant modes of inheritance (Bruce and Bruce, 1978).

Neurospora shows a circadian rhythm in the periodic formation of conidia as the cultures grow along the length of cylindical growth tubes. The *band* strain produces conidia at intervals of 21.5 h in such "race" tubes. Screening of colonies grown from nitrosoguanadine-treated conidia revealed a variety of mutants with altered periods (Feldman and Hoyle, 1973, 1976). Seven of these are single-gene mutants at the same genetic locus, designated "frequency" (*frq*), on linkage group IIIR. Three have periods shorter than normal (down to 18 h) and four have longer periods (up to 29 h). Mutants at the *frq* locus share important properties. Light pulses evoke responses that suggest that their subjective day part of the cycle is altered but their subjective night portion is unaffected. Mutants are incompletely dominant to wild type, as heterokaryons containing mutant and wild-type nuclei have circadian periodicities intermediate in length. The changes in period are proportional to the percentage of mutant nuclei—a gene dose effect. Each mutant differs from wild type in period length by 2.5, 5.0, or 7.5 h, as if alterations in the period occur in discrete steps (Feldman *et al.*, 1979).

While mutations at the *frq* locus of *Neurospora* are mutations of a single gene that does not affect other characteristics (*e.g.*, growth), mutations at five other genetic loci also can alter the period. No unique locus determines period length. Construction of double or triple mutants shows that mutations' effects are often cumulative—one triple mutant had a period length of 38.5 h (Feldman *et al.*, 1979).

A summary of circadian-clock period mutants (Table I) shows that mutations are not consistently dominant or recessive, that they can occur at different loci in the same organism, and they can greatly decrease or increase the period length,

TABLE I

Circadian clock mutants

Organism	Strain	Period	Linkage	Dominance	Reference
Drosophila					Konopka & Benzer (1971)
melanogaster	per^s	19	X chromosome	Incomplete	
	per^l	28	X chromosome	Recessive	
	per^0	aperiodic	X chromosome	Recessive	
D. pseudobscura					Pittendrigh (1974)
	group 1	aperiodic	X chromosome	Incomplete	
	group 2	aperiodic	X chromosome	Incomplete	
Chlamydomonas					Bruce & Bruce (1978)
	per 1	28	—	Dominant	
	per 2	27	—	Recessive	
	per 4	28	—	Incomplete	
Neurospora					Feldman *et al.* (1979)
	frq 1	16.5	VIIR	Incomplete	
	frq 2	19.3	VIIR	Incomplete	
	frq 3	24.0	VIIR	Incomplete	
	frq 4	19.3	VIIR	Incomplete	
	frq 6	19.2	VIIR	—	
	frq 7	29.0	VIIR	Incomplete	
	frq 8	29.0	VIIR	—	
	prd	25.8	IIIC	Recessive	
	chr	23.5	VIL	—	
	IV 2	25.5	V R	—	
	IV 4	25.1	IC	—	
	V 8	18	?	—	

perhaps in discrete steps. The circadian clock mechanism is complicated and appears to require many gene products for proper functioning.

Screening biochemical mutants for clock abnormalities also has been productive. Crossing the *band* strain of *Neurospora* with an oligomycin-resistant strain shortened the normal 21.5 h period to 18–19 h and slowed growth by 30% (Dieckman and Brody, 1980). The oligomycin-resistance locus maps very close to the *frq* clock-period locus. The resistance itself may be due to a change in a single mitochondrial protein. However, the real significance of this finding is yet to be determined, because oligomycin does not phase shift the clock in *Neurospora* and the oligomycin-resistance mutation may include more map units than a single peptide.

Another way that screening biochemical mutants can be useful is shown by a recent result in *Neurospora* (Nakashima *et al.*, 1980, 1981a). Cycloheximide-resistant strains crossed with *band* produced mutants that had normal rhythms but, unlike normal *Neurospora*, could not be phase shifted by cycloheximide. This showed that cycloheximide phase shifts the rhythm by inhibiting protein synthesis, and not by some side-effect of the inhibitor molecule.

Models

A plausible model of the circadian clock, the membrane model (Njus *et al.*, 1974; Njus *et al.*, 1976), brings together much of the biochemical evidence and mathematical descriptions of the circadian oscillator. Two propositions are implicit in the model: (1) the clock is found in a single cell, and (2) all eucaryotes have a clock based on the same principles. The model is compatible with the prevailing

view that the clock is a limit-cycle oscillator (Fig. 9, and Tyson *et al.*, 1976). It identifies the ion gradient and ion transport activity of the cell as the state (dependent) variables in this oscillator. The ion gradient would develop across a membrane (unspecified for lack of evidence) *e.g.* of an organelle, such as the endoplasmic reticulum, or the plasma membrane. The oscillator's trajectory in time is traced out in the phase plane as a stable-limit cycle (Fig. 9) in which the X axis is ion concentration and the Y axis is ion transport activity. Each point on the limit cycle, quantified by a value for each variable, corresponds to a specific phase (or circadian time: 0, 6, 12, 18 h, etc.). Phase shifting could entrain the oscillator to a forcing LD cycle. In the model, light reduces the ion gradient (X axis) instantaneously to a new value dependent upon the strength of the light pulse (*e.g.*, dotted lines at CT 10 and 18). The oscillator would resume motion from that point and eventually would return to the stable limit cycle, but with a permanent phase shift—advanced or delayed depending on the time in the cycle when the pulse was given. The same action would be expected of any agent (*e.g.*, chemical pulse) that altered the ion gradient.

To explain the generation of slow oscillations, suitable for circadian periods, Njus *et al.* suggested that the arrangement of membrane particles determines transport activity, and that rearranging these components was a slow process requiring lateral diffusion in the lipid of the membrane. The adjustment of the saturation of membrane lipids in response to temperature changes was seen as a possible explanation for temperature compensation of the period shown by circadian clocks. Thus, the membrane model offers explanations for most of the features of circadian clocks.

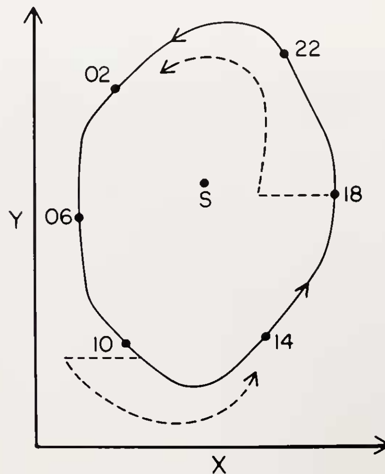

FIGURE 9. Limit cycle oscillator, depicting the relationship between the state variables X and Y in the phase plane. Time (or phase) in the circadian cycle is given by the circadian time points, 02, 06, 10, 14, 18, 22, which are successively encountered with counterclockwise motion around the stable limit cycle (solid line). The state variables in the membrane model are X = ion concentration and Y = ion transport, but other variables could be appropriate in other models. Perturbations of the oscillator with natural time givers or chemical pulses are shown here (CT 10 and CT 18) to reduce the X variable instantly, pushing the oscillator off the limit cycle. The oscillation (dotted lines) then approaches the limit cycle again but with a permanent phase advance (CT 18) or phase delay (CT 10). The amount of shift depends upon the strength of the perturbing stimulus. If the oscillator is driven to the singularity point(s) arrhythmia results until the oscillator is moved off the singularity. Adapted from Njus *et al.*, 1976, and Tyson *et al.*, 1976.

The membrane model is not explicit on many points (*e.g.* which ions, which membranes). But it focuses attention on the evidence for the involvement of ions and ion transport in the clock. High potassium pulses phase shift the rhythms of organisms as diverse as *Aplysia* (Eskin, 1972) and bean plants (Bünning and Moser, 1973). Lithium lengthens the period of rhythms (Engelmann, 1973). The ionophores valinomycin (Sweeney, 1976) and A23187 (Eskin and Corrent, 1977) phase shift rhythms, and ethanol is notorious for its period-lengthening and phase-shifting effects. Sweeney (1976) and Eskin (1979) summarized the evidence for the involvement of ions and membranes in the clock.

Progress in gathering additional evidence to support the membrane model has been slow, and evidence in favor of the involvement of protein synthesis in the clock has accumulated rapidly. This latter evidence will be reviewed in a later section, but it should be noted here that ion gradients and ion transport might be controlled by alternating synthesis and degradation of conductance or transport channel proteins (Njus *et al.*, 1976) or of transport enzymes. Also, membranes may provide a surface for maintaining the proper geometrical arrangement of ribosomal units during protein synthesis (Palade, 1975).

Other models with varying degrees of utility have been proposed. One was the chronon model of Ehret and Trucco (1967), in which messenger RNA would be sequentially transcribed from a clock gene. Scant evidence supports this model. One model offered frequently is a feedback-relaxation oscillator model, exemplified by that of Benson and Jacklet (1977b) for the *Aplysia* eye rhythm. In this model a chemical substance(s) (now thought to be specific proteins) is synthesized and accumulates to a certain level. Further synthesis is controlled by comparing the existing level to a reference level. If the reference level is exceeded, synthesis is switched off, resuming again when the level falls below the reference. Light can adjust the reference level and thereby phase shift the oscillator. This feature is similar to light-induced phase shifting in the membrane model, where light acts to reduce an ion gradient. Blocking synthesis also can cause phase shifts in the model. The phase shifts in the model resulting from light or synthesis inhibition differ, as found experimentally.

The coupled translation-membrane model proposed by Schweiger and Schweiger (1977) features essential membrane proteins (synthesized each cycle) and membrane assembly as the oscillator's central components. The essential proteins, assembled into membranes, change the characteristics of the membranes. Regulation of protein synthesis (switching on and off) depends upon thresholds in the loading of proteins into the membrane. This is analogous to the reference level and switching in the Benson and Jacklet model. Neither of these models offers a concise outline of the oscillator.

A biochemical feedback model involving c-AMP has been proposed by Cummings (1975), but more recent evidence on the involvement of c-AMP does not support this concept: *Neurospora* mutants deficient in adenylate cyclase have perfectly good rhythms (Feldman *et al.*, 1979).

Macromolecular synthesis, proteins, and RNA

Protein synthesis on the eucaryotic ribosome (80S) is now viewed as an important aspect of the circadian timing mechanism, although some early experiments examining this proposition were equivocal (Sweeney *et al.*, 1967). Good evidence supporting the involvement of protein synthesis was obtained by Feldman (1967), who showed lengthening of the period of the biological clock in *Euglena* by step

applications of cycloheximide, an inhibitor of protein synthesis. The effect was proportional to the degree of inhibition of protein synthesis. This work stood alone for several years before other positive results were obtained.

According to the criteria discussed at the Dahlem Conference (Tyson *et al.*, 1976), a chemical agent, such as a protein synthesis inhibitor, affects the basic clock mechanism if the period of the clock is altered by the continuous presence of the agent or if the phase of the rhythm is shifted when the agent is applied as a brief pulse (*i.e.*, short compared to the length of the cycle, *e.g.*, 4–6 h). The continuous application, or step experiment, is believed to affect a parameter of the clock such as the rate of substrate utilization. The step experiment should give results proportional to the dose of the chemical being tested. A threshold concentration should be apparent. The pulse experiments are thought to act on a state variable (dependent variable that describes the oscillation) if phase shifts of the rhythm are obtained. Again, a threshold concentration should be definable and the size of the phase shift should be dose dependent. The size and direction of the phase shift also should depend upon the phase of the cycle when the pulse was given—that is, phase shifts should be phase dependent.

In an ideal situation, where the parameters and state variables of an oscillator are known, one should be able to accurately predict the effects of chemicals used in step and pulse experiments. However, the parameters and state variables of the circadian oscillator are unknown, and any distinction between parameters, state variables, or just hands of the clock is model-dependent. For example, a simple model may have only two state variables, such as ion concentration and ion transport in the membrane model. A more complete description might include additional state variables. But despite such uncertainties about their interpretation, the step and pulse experiments remain good approaches to probing the clock mechanism.

Most studies of the oscillator's protein synthesis requirement have used inhibitors of protein synthesis in step and pulse experiments. Both experiments have been made on the *Aplysia* eye rhythm. The results serve as examples here. Anisomycin, a potent protein-synthesis inhibitor that binds to the 60S subunit of the eucaryotic ribosome (Grollman, 1967), was applied in increasing dosage in a step experiment (Jacklet, 1980a). The results are shown in Figure 10. The threshold concentration for lengthening the period of the rhythm is about 10^{-8} *M*. This concentration inhibits protein synthesis by about 10% (Grollman, 1967). At 10^{-7} *M* (inhibition about 50%) the period is lengthened to more than 30 h. At 10^{-6} *M* (about 90% inhibition) the rhythm is immediately suppressed; the CAP activity is continuous, but lacks its circadian periodicity. This is a specific effect on the clock control, as the anisomycin does not seem to affect the CAP-generating mechanism. Lack of effect on neuronal membrane function was independently shown by Schwartz *et al.* (1971).

Pulses of anisomycin at 10^{-6} *M* permanently phase shift *Aplysia* eye rhythm, as shown in Figure 11. Delays (top panel) or advances (lower) are obtained depending upon the phase of the rhythm at which the pulses are applied. The rhythm returns to its normal periodicity after the pulse, showing that the inhibitor's effects on the clock are reversible, as are the effects of anisomycin on protein synthesis (Grollman, 1967). Transient differences in the amount of the phase shift often occur in the first cycle after the pulse, but a stable permanent shift is established by the third cycle. These differences were particularly noticeable at some circadian phases, showing a differential sensitivity of the oscillator to the perturbation.

Applying anisomycin pulses at different phases of the circadian cycle causes systematic differences in the direction and magnitude of the phase shifts, just as

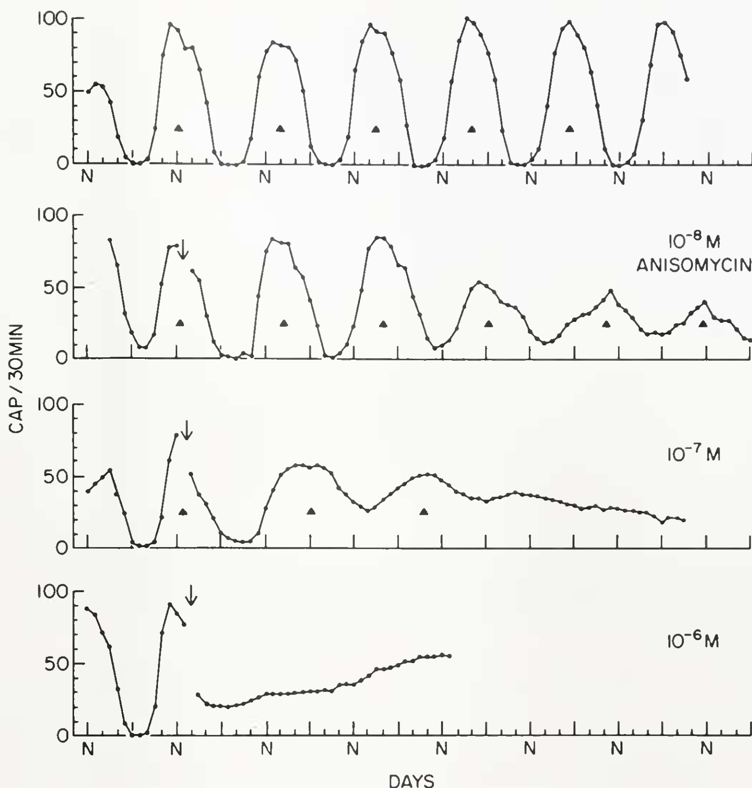

FIGURE 10. CAP frequency rhythms of *Aplysia* during the continuous presence, added at arrows, of anisomycin at 3 concentrations. The top is a control eye at 15°C in darkness, exhibiting periods of 26.5, 26.5, 26.8, 27, and 27 h. Periods at 10^{-8} *M* are 27.5, 27.5, 28.5, 32, and 26.5. Periods at 10^{-7} *M* are 35 and 31 h. The inhibitor suppressed the rhythm completely at 10^{-6} *M*, but the CAP activity continued. Successive noons (N) are the time axis. Triangles are calculated centroid points. From Jacklet, 1980b.

is expected for perturbations of a state variable (Tyson *et al.*, 1976). The phase shifts plotted against circadian time in the cycle resulted in a PRC that indicates the agent's action on the basic timing mechanism. Figure 12 shows the PRC for anisomycin pulses. Greatest delays are at CT 0–3; advances are at CT 5–7. This apparent change in sensitivity to an inhibitor suggests that proteins necessary for clock timing are synthesized at specific times (Karakashian and Schweiger, 1976b). However, this proposition has not been tested, as it is not known which newly synthesized proteins are involved in the clock mechanism and which ones might be driven by the clock. Thus, a resolution must await the identification of specific clock proteins.

In addition to *Euglena* and *Aplysia* eye, other preparations have supplied strong evidence to support involvement of protein synthesis in the oscillator. The rhythm in *Acetabularia* is phase shifted by three inhibitors: puromycin, cycloheximide, and anisomycin (Karakashian and Schweiger, 1976b). Using a liquid culture technique for *Neurospora*, Nakashima *et al.* (1980, 1981b) demonstrated phase-dependent phase shifting with cycloheximide. Even in *Gonyaulax*, where such experiments are difficult, there is positive evidence for phase shifting with cycloheximide (Walz

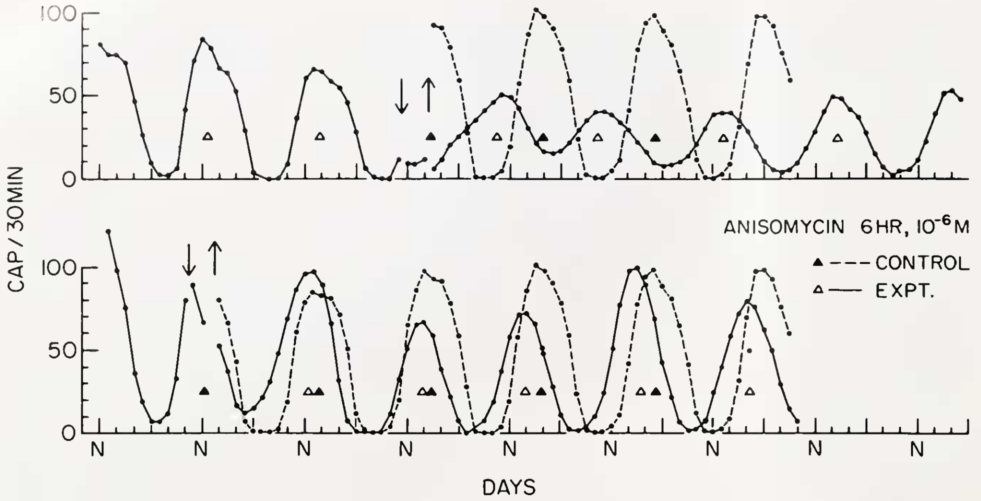

FIGURE 11. Phase shifts of the CAP frequency rhythms of *Aplysia* by 6 h pulses of anisomycin (10^{-6} *M*). The upper graph shows a phase delay when the pulse was given at CT 3 and the bottom graph shows a phase advance when the pulse was given at CT 5. Solid lines are experiment eyes and dotted lines are controls. Triangles are calculated centroid points. From Jacklet, 1980b.

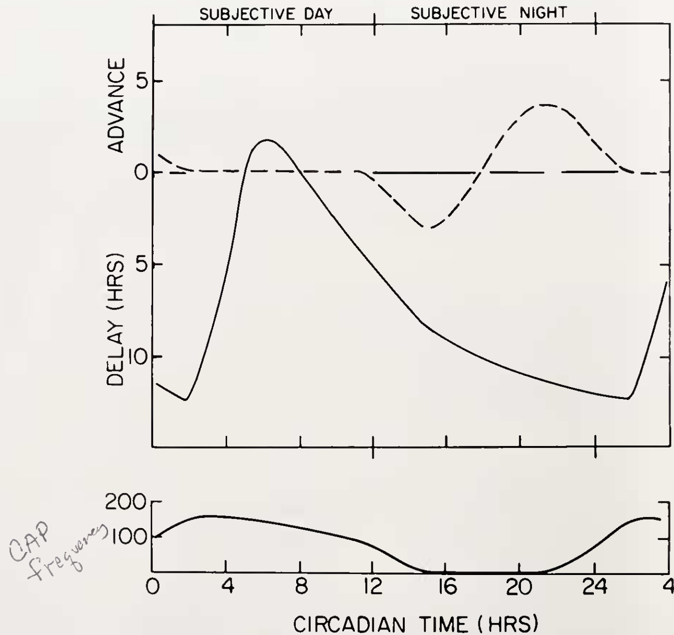

FIGURE 12. Phase response curves (PRC) for anisomycin (solid lines) and light (dotted lines) for the *Aplysia* eye rhythm. The time scale is circadian time divided into subjective day (00–12) and subjective night (12–24). The transition from phase delays to phase advances occurs about CT 18 for light pulses but at about CT 5 for anisomycin. The bottom of the figure shows the CAP frequency rhythm for phase reference. Adapted from Jacklet, 1977 and 1978.

and Sweeney, 1979; Dunlap *et al.*, 1980). The rhythm of ultrastructural changes in these cells also was phase shifted by cycloheximide (Rensing *et al.*, 1980).

Where the PRC has been extensively studied, it appears to change in a regular way in response to protein synthesis inhibitors at different concentrations. Phase shifts are smaller and have different shapes with lower concentrations. But the position of the PRC on the circadian time axis is relatively fixed (Walz and Sweeney, 1979; Dunlap *et al.*, 1980). This is also true of light-induced phase shifts. Insight into the clock may be gained from similarities and differences in PRCs evoked by various agents. The anisomycin PRC and the light PRC in *Aplysia* clearly differ, as shown in Figure 12. One may conclude that light and anisomycin act on different phases and probably different components of the clock mechanism. This prediction, indeed, follows from the model of Benson and Jacklet (1977b), where the phase-dependent effects of light, which acts on a reference level, should be different from the effects of anisomycin, which acts on synthesis.

However, in the *Gonyaulax* rhythm, the inhibitor cycloheximide may have two actions, an early one on ion distribution and a later one on protein synthesis (Walz and Sweeney, 1979). Also, the PRC is nearly identical to the PRC for light pulses. This points out the dangers of generalizing from one organism to others.

Other protein synthesis inhibitors, such as cycloheximide and puromycin, cause phase shifts of the *Aplysia* eye clock (Rothman and Strumwasser, 1976). The PRC they constructed for puromycin was similar to the one for anisomycin. These puromycin experiments were repeated using an organ culture procedure. They yielded a PRC nearly identical in shape and time position (but smaller in magnitude) to the anisomycin PRC (Lotshaw and Jacklet, 1980). This supports the notion that similar inhibitors should cause similar clock perturbations. Some other agents provoke PRCs in *Aplysia* similar to the anisomycin PRC. These include the metabolic inhibitors NaCN, DNP, the calcium ionophore A23187 (Eskin and Corrent, 1977); and low temperature (Deuser, 1979; Benson and Jacklet, 1977a) which yield PRCs with similar time positions but smaller magnitudes. The general metabolic inhibitors seem to mimic the more specific action of protein synthesis inhibitors, which act on a synthetic process that has high energy demands.

Inhibitors may have side effects. Therefore, it is important to show that any side effects are minimal and that protein synthesis is actually inhibited at the inhibitor concentration where clock effects are observed. In the *Acetabularia* experiments (Karakashian and Schweiger, 1976a) protein synthesis was about 50% inhibited at the concentration effective for phase shifting. In the *Neurospora* experiments (Nakashima *et al.*, 1980, 1981b) synthesis was reduced by 65%. In *Aplysia* 50% inhibition by puromycin was effective in phase shifting (Rothman and Strumwasser, 1976) and 80–90% inhibition with anisomycin was observed at the concentration (10^{-6} *M*) that affected phase-shifting (Jacklet, 1977). Thus, in a good proportion of the tests, the concentration of inhibitor that causes 50% or more inhibition also produces phase shifts of the circadian clock. Inhibitors of procaryotic protein synthesis are uniformly without effect on the clock (Karakashian and Schweiger, 1976b), implicating the eucaryotic ribosome as an important part of the clock. This is consistent with the lack of circadian rhythms in procaryotes.

However, inhibition and phase shifting could be parallel events, not necessarily causally linked. Side effects as well as protein-synthesis inhibition might be dose dependent. Two experiments strongly support a causal link between protein-synthesis inhibition and phase shifting the circadian clock. In the first, Jacklet (1980b) tested derivatives of the inhibitor anisomycin that are very similar to the parent

molecule but do not inhibit protein synthesis. Only the active inhibitor molecules caused phase shifts. The inactive derivatives were completely innocuous. In the second experiment, Nakashima *et al.* (1980, 1981a), used cycloheximide-resistant *Neurospora* mutants. They demonstrated that cycloheximide is ineffective in phase-shifting those mutants, but quite effective on wild-type *Neurospora.* Therefore, the insensitivity of the ribosomes of the mutants to cycloheximide confers immunity to the inhibitor and prevents phase shifting.

Karakashian and Schweiger (1976c) noted in *Acetabularia* a difference in cycloheximide sensitivity at different temperatures. The sensitivity shifted to a different circadian phase at a different (20° vs. 25°C) physiological temperature. Since the clock is temperature compensated, this unexpected result suggested that protein synthesis was independent of the central clock mechanism. This inconsistency remains for *Acetabularia.* However, tests of other rhythms showed that the effects of inhibitors are nearly identical at different physiological temperatures, and therefore the effect is temperature compensated, in *Aplysia* (Jacklet, 1980a), *Gonyaulax* (Dunlap *et al.*, 1980), and *Neurospora* (Nakashima *et al.*, 1980).

If protein synthesis is important, then RNA synthesis could be, too, since DNA codes for RNA and RNA codes for protein. The importance of DNA synthesis has been tested in *Gonyaulax* by applying the inhibitor mitomycin C (Karakashian and Hastings, 1963). This had no effect on the circadian clock. RNA synthesis was tested using actinomycin D. It had weak effects on phase shifting the *Gonyaulax* glow rhythm and blocked rhythmicity in *Acetabularia* (Sweeney *et al.*, 1967) and in the *Aplysia* eye (Rothman and Strumwasser, 1977). The strongest evidence against RNA synthesis in clock timing is the observation of strong rhythms in enucleated *Acetabularia* (Sweeney and Haxo, 1961) and the failure of rifampicin (Vanden Driessche *et al.*, 1970) to block the rhythm in enucleated cells.

Ionizing radiation (X-rays) has been used to selectively block the circadian rhythm in the *Aplysia* eye, without altering membrane functions (Woolum and Strumwasser, 1980). Their results suggest that the eye contains a number of circadian oscillators, most of them near the optic nerve. The most likely targets of the X-rays, and therefore the elements of importance for the circadian clock, are nucleic acids. The appearance of the eye activity after irradiation is similar to the activity shown in Figure 10 after step treatment with 10^{-6} *M* anisomycin.

Most of the RNA-synthesis inhibitor studies suffer from a lack of actual measurements of the RNA synthesis. Side effects of the inhibitors may be suspect. Also, the drugs used, such as antinomycin D, are not readily reversible, making it impossible to study the periodicity or phase shift of the rhythm after treatment. A reversible RNA synthesis inhibitor with minimal side effects, and measurement of the actual RNA synthesis rates, are needed to completely resolve whether RNA synthesis is involved in the circadian clock. The evidence to date favors the conclusion that RNA synthesis is not part of the basic clock (Sargent *et al.*, 1976), but it seems likely that the amount of RNA available could readily influence the clock timing generated by protein synthesis on the eucaryotic ribosome.

Acknowledgment

This work was supported in part by National Science Foundation grant BNS 11154. I thank Alice Jacklet for helpful comments, Thea Hotaling for assistance, and Robert Speck for figure production.

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